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ÉCOLE POLYTECHNIQUE FÉDÉRALE DE LAUSANNE
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6723373003
SWITZERLAND

Patents ADP number (*if you know it*)

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

CHEMICAL ASSAY APPARATUS

5. Name of your agent (*if you have one*)

"Address for service" in the United Kingdom to which all correspondence should be sent
(*including the postcode*)

J.Y. & G.W. JOHNSON,
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WC1V 7DP

Patents ADP number (*if you know it*)

976001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (*if you know it*) the or each application number

Country	Priority application number (<i>if you know it</i>)	Date of filing (<i>day / month / year</i>)
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7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing (<i>day / month / year</i>)
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8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if:

- a) any applicant named in part 3 is not an inventor, or
- b) there is an inventor who is not named as an applicant, or
- c) any named applicant is a corporate body.

See note (d))

YES

CHEMICAL ASSAY APPARATUS

This invention relates to apparatus for detecting the presence of a target species in an aqueous sample, and also to apparatus for determining the concentration and reaction kinetics of target species. The invention is applicable to the monitoring of many different molecular interactions, in particular molecular recognition between an immobilised affinity partner and a species in solution, such as immunoglobulin/antigen interaction, DNA hybridisation, haptamer-protein interaction, drug and virus detection and high throughput screening of synthetic molecules.

As many affinity complexations between two reaction partners are diffusion controlled, the time needed to reach reaction equilibrium is directly dependent on the mass transport of the molecule. The diffusion time of a molecule in a solution is proportional to the square of the path length; typically a small molecule needs less than one second to diffuse through 10 μm while it needs two hours to traverse one millimetre. In order to decrease the equilibrium time of the reaction, the chemical partners must therefore be placed as close as possible to each other; by reducing the reactor size to microdimensions, immobilising one partner on the surface of the reactor and filling the reactor with the second partner, the equilibrium time can be dramatically decreased.

The use of microreactors not only enhances the speed of affinity assays, but also facilitates the obtaining of information concerning reaction kinetics, which is important in the understanding of the thermodynamic stability of complexes. The affinity constant K_d is the ratio between the forward and reverse reaction rate constants k_+ and k_- , which represent the association and the dissociation constants respectively. A strong complexation is characterised by a very fast association and a very slow dissociation, which in the particular case of sorbent affinity assays are adsorption and desorption from the

These miniaturised systems have been termed "microscale total analysis systems" (μ -TAS)¹, and they have already been recognised as convenient means of manipulating and analysing small sample quantities²⁻⁸. Most μ -TAS devices to date 5 have been produced by photolithography, wet chemical etching or thin film deposition on substrates such as glass, quartz and silicon^{9, 10}. In order to decrease production costs, plastic substrates have also been micromachined using either silicone rubber casting¹¹⁻¹⁴ injection moulding¹⁵ embossing 10^{16, 17} or laser photoablation¹⁸. These structures are planar devices with channels of micrometre size that are often sealed by thermal or anodic bonding to a glass cover. Interconnected channels may be fabricated easily, which makes possible the rapid separation and reactions in volumes 15 of few a picoliters. Other advantages of μ -TAS are the reduction of sample and reagent consumption and the increase of precision and reproducibility relative to bench scale apparatus^{21, 22}.

Competitive immunoassays have also been performed on 20 micro-chips²³⁻²⁵, but the micro-channels have only been used to electrophoretically separate free and bound forms of antigen or antibody. In these assays, antibody and labelled antigen are added in specific quantities to the sample to be analysed. The sample is then incubated with a 25 mixture of the labelled and native antigens that compete for a limited number of antibody binding sites. The micro-channel is then used to separate the free labelled antigen from the complex by capillary electrophoresis, and quantitation is performed by luminescence (fluorescence or 30 chemiluminescence) at the end of the separation channel. The amount of free labelled antigen measured is then related to the analyte concentration in the sample using a previously determined calibration curve. In this type of assay it is essential to avoid adsorption of a reaction partner on the 35 micro-channel walls.

Another type of immunoassay device has been developed for simultaneous analysis of multiple samples²⁶. In this

and other proteins by such methods. In clinical diagnostics for instance, competitive and sandwich immunoassays using luminescence detection are now used on a routine basis ^{27, 28}.

In enzyme-mediated immunoassays, a molecule is labelled 5 with an enzyme that catalyzes the luminescence reaction. Typical examples are the detection of immunoreagents labelled with Horse Radish Peroxidase (HRP) or Alkaline Phosphatase (ALP) which, in the presence of hydrogen peroxide and hydroxide ions, respectively facilitate the 10 oxidation of luminol and dioxetanes and the hydrolysis of phosphate-containing reagents. Similarly, ALP has been used in CF assays to cleave a phosphate group from a fluorogenic substrate to yield a highly fluorescent product ²⁹.

Luminescence assay methods are widely used in the 15 analysis of peptides, proteins, and nucleic acids. CL has been shown to be a highly sensitive detection method in both flow injection analysis (FIA) and high-performance liquid chromatography ³⁰⁻³², and it has also been employed in capillary electrophoresis (CE) ^{33, 34} for the detection of 20 amino acids neurotransmitters ³⁵, rare-earth metal ions ³⁶ or labelled proteins ³⁷. However, it is in immunoassays that luminescence is the most commonly used detection method ^{27, 34, 38-43}.

Among prior art methods for the measurement of 25 enzymatic reaction rate, US 4,621,059 discloses a method in which the light emitted by a luminescent substance flowing through a capillary column and reacting with an immobilised enzyme is collected through a plurality of optical fibers that are arranged along the longitudinal direction of the 30 column in order to determine the enzyme activity or the quantity of analyte of interest from the distribution of the luminescence intensity.

US 5,624,850 describes a method for performing immunoassays in capillaries in which fluorescence is used to 35 detect an analyte of interest in translucent capillaries

plurality of microchannels may be served by a single inflow channel, feeding into a common conduit communicating with the microchannels. In some preferred embodiments, the fluid entry force is provided by aspiration means connected to a 5 common conduit communicating with each microchannel at its end distal the inflow channel. In other embodiments, the apparatus comprises a rotatable support member and the fluid entry force is provided by centrifugal force upon rotation of the substrate. Conveniently, the support member may form 10 the substrate of the microchannel apparatus, with the microchannels being arranged generally radially. Alternatively the rotatable support member may serve as a support for one or more devices having parallel microchannels.

15 The advantage of a common source of fluid entry force for all of the microchannels is that simultaneous filling may be ensured, the fluid samples being prevented from entering the microchannels by the hydrophobic gate means until the fluid entry force is applied. Furthermore, the 20 degree of fluid entry force may also readily be controlled, to ensure rapid filling of the microchannels, and adequate mixing. The microchannels may also be emptied in an efficient and rapid manner, by application of an increased force to the fluid in the channels, for example by 25 increasing the degree of aspiration, or by increasing the rate of rotation of the rotation support member. An exact end point of an assay may thereby be achieved. In many instances it is advantageous for the sample to be expelled before monitoring for bound target species.

30 If desired, a liquid reagent or a washing fluid may be supplied in a sealed cavity forming a reservoir, there preferably being one such reservoir per microchannel. The reservoirs may be arranged to communicate with their respective microchannels via normally closed valves, and may 35 be caused to expel their contents through such valves when acted upon by respective pistons. Alternatively, there may

the reaction chamber(s), at least a portion of the or each fluid inflow channel having a hydrophobic inner surface adapted to act as gate means to prevent passage of fluid through the fluid inflow channel into the reaction 5 chamber(s) until such fluid is acted upon by a fluid entry force.

For ease of fabrication, the apparatus is preferably formed in two main parts: a substrate in which the microchannels (and possibly also the inflow channels) are 10 formed as depressions (for example by injection moulding, hot embossing, photoablation, casting or polymerisation on a mould); and an overlying layer applied over the substrate and over the depressions, to form the microchannels (and optionally also the inflow channels). In embodiments in 15 which the inflow channels are not produced in the substrate they may, for example, be produced by drilling through a laminated overlying layer using a laser, or by depositing above the inlet of the reaction chamber a joint made of a hydrophobic material such as polydimethylsiloxane (PDMS).

20 The apparatus may be formed from any suitable material, for example, ceramics, glass, semiconductors, polymers, or combinations thereof. In a particularly preferred embodiment, both the substrate and lamination layer are formed of polymer material, which not only permits ready 25 formation of the microchannels (for example by photoablation), but also allows the two components to be fused together by a thermal lamination technique. For this purpose, it is preferred that at least one of the polymers is of a material which has a relatively low melting point, 30 for example polyethylene with a melting point of under 200°C. The lamination layer may with advantage be of an elastomeric material, such as polydimethyl siloxane (PDMS). In apparatus for use in conjunction with optical detection 35 means, it is preferred that the lamination layer be formed of a substantially transparent material, and the substrate of a substantially opaque material (such as a ceramics material or a carbon-filled polymer).

Fig 2 is a schematic plan view of an embodiment of apparatus according to the invention, illustrating the steps of parallel sampling, loading and washing, achieved by aspiration;

5 Fig 3 is a schematic plan view of an alternative embodiment of apparatus according to the invention, in which parallel filling and washing step are achieved by centrifugal force;

10 Fig 4 is a schematic plan view of a further embodiment of apparatus according to the invention, in which the solution is loaded by slight aspiration and washed by strong aspiration;

15 Fig 5a is a partial plan view of an embodiment of apparatus according to the invention, incorporating a fluid reservoir adjacent the fluid inflow channel;

Fig 5b consists of two vertical cross sections of the Fig. 5a apparatus together with an associated piston, illustrating the action of the piston in penetrating the sealed reservoir and expelling its contents through a valve 20 into the reaction chamber;

Fig 6 is a top plan representation of an embodiment of apparatus according to the invention manufactured by UV-Laser photoablation of a polycarbonate compact disk, the embodiment being constructed substantially as the apparatus 25 of Fig 3;

Fig 7 is a calibration curve obtained from a fluorescence imager using ALP-DDi solution in the microchannel of an embodiment of apparatus according to the invention;

30 Fig 8 is a graph illustrating fluorescence results indicating the level of binding between DDi-ALP in a test involving use of two microchannels of apparatus according to

linear channels 22 mm long. The microchannels are typically between 1 and 1,000 μm in width, and in this example are approximately 100 μm wide. The depth of the channels was fixed at 40 μm , by controlling the number of laser pulses used (each pulse photoablates approximately 150 nm). The channels are then sealed by thermal lamination of a layer of polyethylene over the base polymer sheet, the channels then exhibiting a trapezoidal shape in which three walls are composed of the substrate polymer (PET or Polycarbonate) and the top is composed of the lamination (Polyethylene). Fluid inflow channels (or "gates") (1) are opened either by firing enough laser pulses or are mechanically drilled through the hydrophobic lamination layer. The gates, which may have a diameter between 10 μm and 10mm, have hydrophobic inner surfaces due to the nature of the polymer, and therefore inhibit passage of aqueous fluids.

The precise arrangement of microchannels is not crucial to the operation of the invention, though two general geometries have been developed and tested by the applicants and proved to be of benefit. In the first of these, a plurality of microchannels are arranged parallel to each other, conveniently on a generally rectangular substrate. The inflow channel "gates" of the various microchannels are aligned with each other, to permit rapid and efficient loading with test solutions from a linear multiple pipette device (see Fig 2). In the second configuration, the microchannels are arranged radially on a generally circular substrate, either with the inflow channel gates towards the centre of the circle and the opposite (outflow) ends of the microchannels towards the circumference (Figs 3 and 6), or vice versa (Fig 4).

A number of different means may be employed to provide the fluid entry force, of which the preferred means are aspiration and centrifugal force. In the apparatus of Fig 2, a common conduit (3) is supplied at the outflow ends of the microchannels (2), to which a reduced pressure is applied during operation of the device, to draw fluid into

movement of piston (11) within reservoir (10) increases the fluid pressure within the reservoir, thus opening valve (12) and allowing fluid from the reservoir to enter the microchannel. Depending upon the requirements of any 5 particular assay, the reservoir may either be filled with a reagent or with wash fluid.

By way of example, various tests were carried out to establish the utility of apparatus according to the invention in performing an immunoassay for D-Dimer. D-Dimer 10 is used as a diagnostic indicator in thromboembolic events: deep vein thrombosis and pulmonary embolism can be diagnosed by monitoring D-Dimer concentration in blood. In the past, the most reliable assay of D-Dimer have been performed by ELISA techniques, for example the "Asserachrom D-Di" of 15 Diagnostics Stago. However, standard ELISA techniques are not suited for emergency situations, and alternative membrane-based techniques have been developed which use colour based detection systems ⁴⁸. However, these suffer from the disadvantage that the detection mechanism is too 20 subjective.

In the present tests, the detection of the enzyme was effected by a chemifluorescent substrate solution (VCR, Amersham). This system is based on the fluorescent detection of the AttoPhos substrate hydrolysed by ALP. The 25 microchannels were then exposed to a Fluorescence Imager screen (MP840, Molecular Dynamics) and every channel was read for 1 minute. The image was then quantified using Image Quant software (Molecular Dynamics). The calibration of the enzyme in the microchannel was achieved by mixing the 30 substrate solution with different concentrations of enzyme and incubating for 5 minutes. The microchannels were then filled with the mixtures and analysed with the fluorescence imager. In the actual tests, the enzyme was immobilised on the surface of the microchannels, and the VCR solution was 35 added to the channels with fluorescence being measured 5 minutes later.

antibodies. This experiment shows that some of the adsorbed antibodies are still active on the surface and that BSA is an effective blocker against the non-specific adsorption of the DDi-ALP complex.

5 Figure 9 shows the fluorescence of the substrate in the channels after adsorption of different concentrations of ALP-DDi on the $10 \mu\text{g.ml}^{-1}$ adsorbed antibodies. The fluorescence intensity of the microchannel lines clearly shows the gradient of concentration in the different 10 microchannels. The relative intensity of every microchannel is shown graphically in Figure 10. Saturation of channels is reached at about $30 \mu\text{g/ml}$.

Figure 11 shows the fluorescence intensity of microchannels that have been incubated for different periods 15 of time. For short incubation times (<5 min), the intensity grows linearly, showing that the antigens are very quickly captured by the antibodies. It is thought that all the antigens have still not reached the surface by diffusion. This first slope approximately follows the diffusion of the 20 molecules to the walls. The molecules then react rapidly and the reaction becomes quasi diffusion-controlled. After 5 minutes of incubation, the reaction is controlled by slower kinetics driven by two different phenomena. Firstly, large 25 molecules diffuse much more slowly and therefore reach the surface after a long time. In this case, the molecules can be partially degraded fibrin products, of which the molecular weight can be larger than 1000 kD. Secondly, there 30 is a tendency for non-specific adsorption; such reactions are much slower than immunological recognition and are driven by electrostatic or hydrophobic interactions that require reorganisation at the molecular level. This type of non specific adsorption may therefore be excluded by short incubation times.

Figure 12 shows the fluorescence dependence of the D-Dimer concentration after a competitive immunoassay. In the 35 low concentration range, most of the immobilised antibody

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8. Apparatus according to any preceding claim, comprising a plurality of separate reaction chambers, each communicating with an inflow channel and associated gate means.

5 9. Apparatus according to claim 8, wherein each reaction chamber is provided with a separate inflow channel.

10. Apparatus according to claim 8, wherein there is one inflow channel forming a common conduit to all reaction chambers.

10 11. Apparatus according to claims 8, 9 or 10, as appendant to claim 2, wherein each microchannel communicates, at its end distal the inflow channel, with a common conduit, the common conduit being connected to aspiration means adapted selectively to apply reduced pressure to the conduit and thus to draw fluid through the 15 microchannel in operation.

12. Apparatus according to claim 10 or claim 11, wherein the common conduit has a cross-sectional area in the range 0.01mm^2 to 25cm^2 .

20 13. Apparatus according to any preceding claim, wherein the microchannels are arranged generally parallel to each other.

14. Apparatus according to claim 13, as appendant to any of claims 10 to 12, wherein the microchannels are 25 arranged generally perpendicularly to the common conduit.

15. Apparatus comprising a plurality of apparatuses according to claim 14 mounted together on a tape.

16. Apparatus according to claim 11, comprising a substantially circular substrate the microchannels being 30 arranged substantially radially, each having its inflow channel towards the circumference of the circle and its

breakable seal, which, in operation, is broken by the piston, wherein movement of the piston into the cavity provides the increased pressure necessary to urge the aqueous fluid from the cavity via the valve into the 5 reaction chamber.

24. Apparatus according to any preceding claim, wherein at least a portion of the surface of the reaction chamber is formed of an electrically conductive material, and the apparatus further comprises electrical detection 10 circuitry connected to said conductive portion, to enable detection of a target species within the reaction chamber by electrochemical means.

25. Apparatus according to claim 24, wherein said conductive portion is formed of a conductive polymer 15 material.

26. Apparatus according to claim 24, wherein said conductive portion is formed by an electrode.

27. Apparatus according to claim 26, wherein said electrode is of a semiconductor material.

20 28. Apparatus according to claim 27, wherein said semiconductor material is substantially transparent.

29. Apparatus according to claim 28, wherein the semiconductor material is indium oxide.

30. Apparatus according to any of claims 1 to 23, 25 further comprising electromagnetic radiation detection means adapted to detect radiation emitted by a target species in the reaction chamber.

31. Apparatus according to claim 30, wherein the detection means comprises at least one photodiode or at 30 least one photomultiplier array arranged along at least a portion of the reaction chamber.

reaction chamber and/or the fluid inflow channel being sealed by an overlying layer applied over the substrate.

41. Apparatus according to claim 40, wherein the substrate and overlying layer are formed of polymer materials, the melting point of at least one of the materials being sufficiently low to permit the substrate and overlying layer to be sealed together by thermal lamination.

42. Apparatus according to claim 41, wherein said at least one material is polyethylene.

10 43. Apparatus according to claim 40 or claim 41, wherein the overlying layer is formed of an elastomeric material.

44. Apparatus according to claim 43, wherein the elastomeric material is polydimethylsiloxane (PDMS).

15 45. Apparatus according to claim 40, as appendant to claim 30 or 31, wherein at least a portion of the substrate is formed of a substantially opaque material and the overlying layer is formed of a substantially transparent material.

20 46. Apparatus according to claim 45, wherein the substantially opaque material comprises a carbon-filled polymer or a ceramics material.

47. A method of manufacturing an apparatus according to any of claims 1 to 46, comprising the following steps which may be performed in either order or simultaneously: forming at least one reaction chamber; and forming at least one fluid inflow channel communicating with the reaction chamber(s), at least a portion of the or each fluid inflow channel having a hydrophobic inner surface adapted to act as gate means to prevent passage of fluid through the fluid inflow channel into the reaction chamber until such fluid is acted upon by a fluid entry force.

55. A method according to claims 53 or 54, wherein the or each sample is applied by means of a pipette, a syringe, or an electrically operated injector.

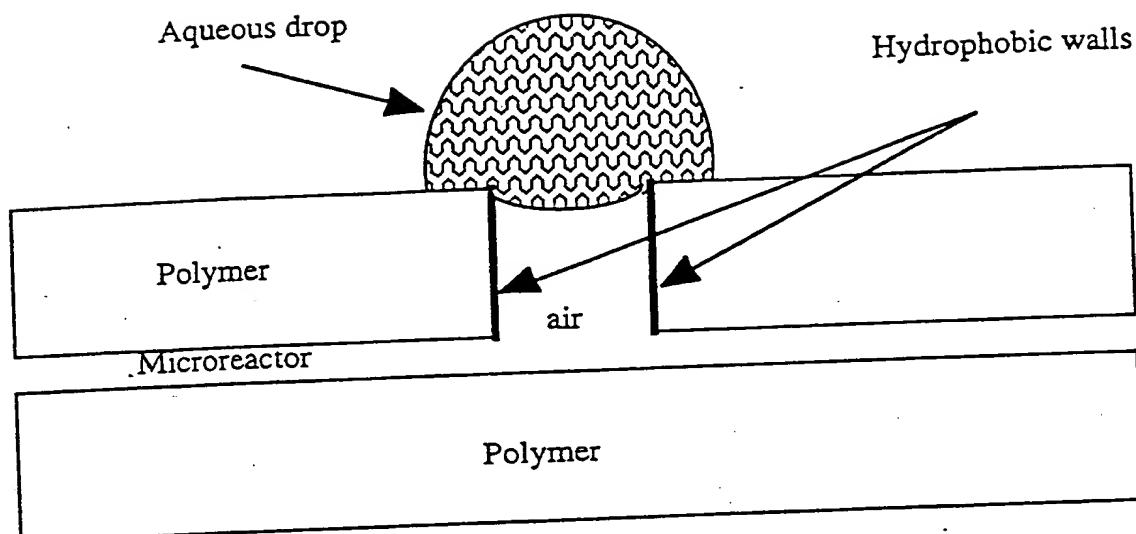
56. A method according to any of claims 53 to 55, for 5 operating an apparatus according to claim 11 or any claim appendant thereto, wherein the fluid entry force is provided by aspiration means, the aspiration means being activated to apply reduced pressure to the or each reaction chamber for a period of time in the range 0.1 to 100s.

10 57. A method according to any of claims 53 to 55, for operating an apparatus according to either claim 19 or claim 15 20, or any claim appendant thereto, wherein the fluid entry force is provided by spinning the substrate or the support member at an angular velocity in the range 1 to 1,000 revolutions per minute for a period of time in the range 1 to 100s.

58. A method according to claim 57, wherein the sample is expelled from the reaction chamber by spinning the substrate at a greater angular velocity, in the range 10 to 20 100,000 revolutions per minute, for a period of time in the range 1 to 100s.

59. A method according to any of claims 53 to 55, for operating an apparatus according to claim 7 or any claim appendant thereto, wherein the fluid entry force is provided 25 by piston pressure.

a)



b)

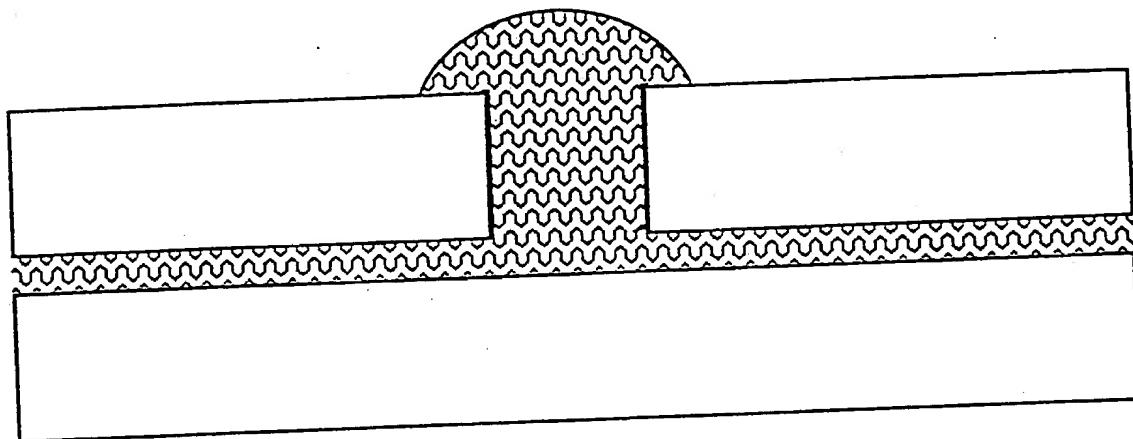


FIG 1

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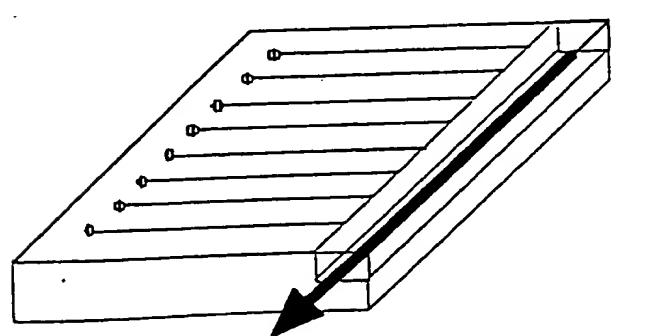
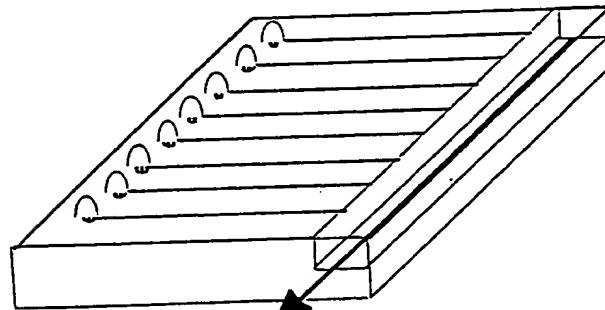
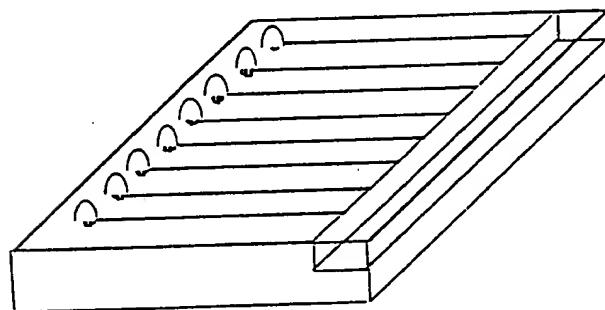
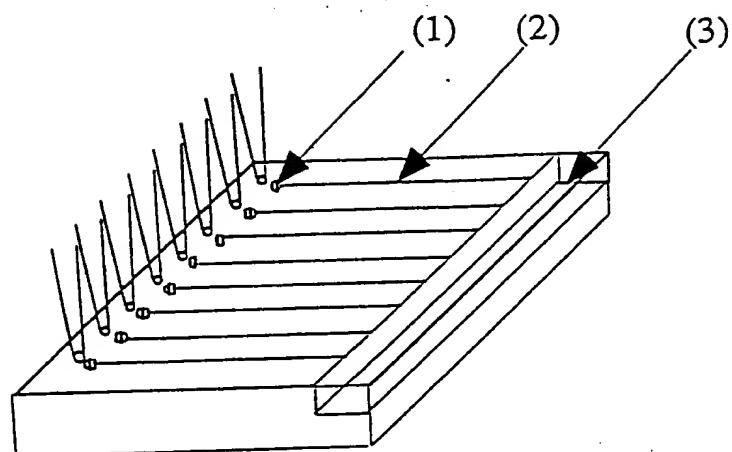


FIG 2

Washing by strong aspiration

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sample dispensing with a pipette

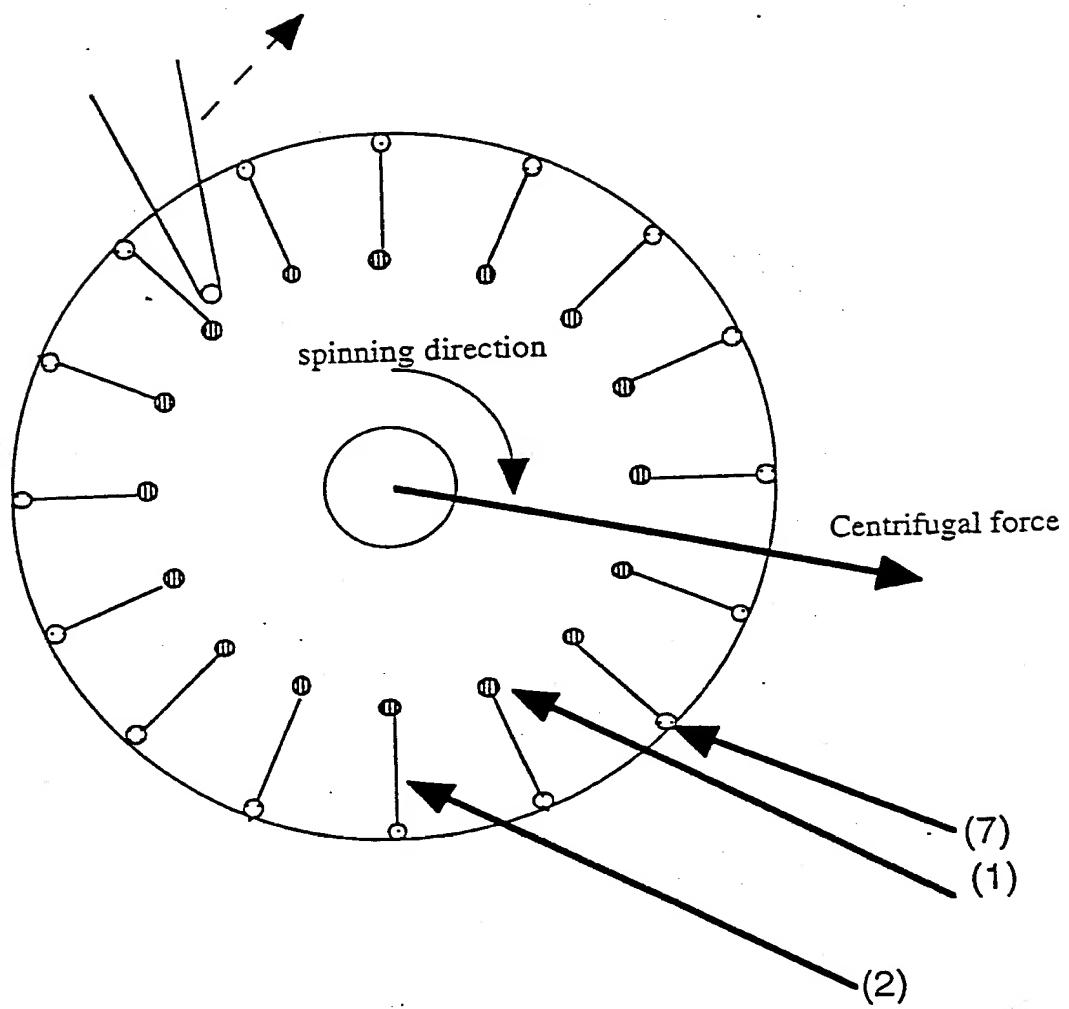


FIG 3

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sample dispensing with a pipette

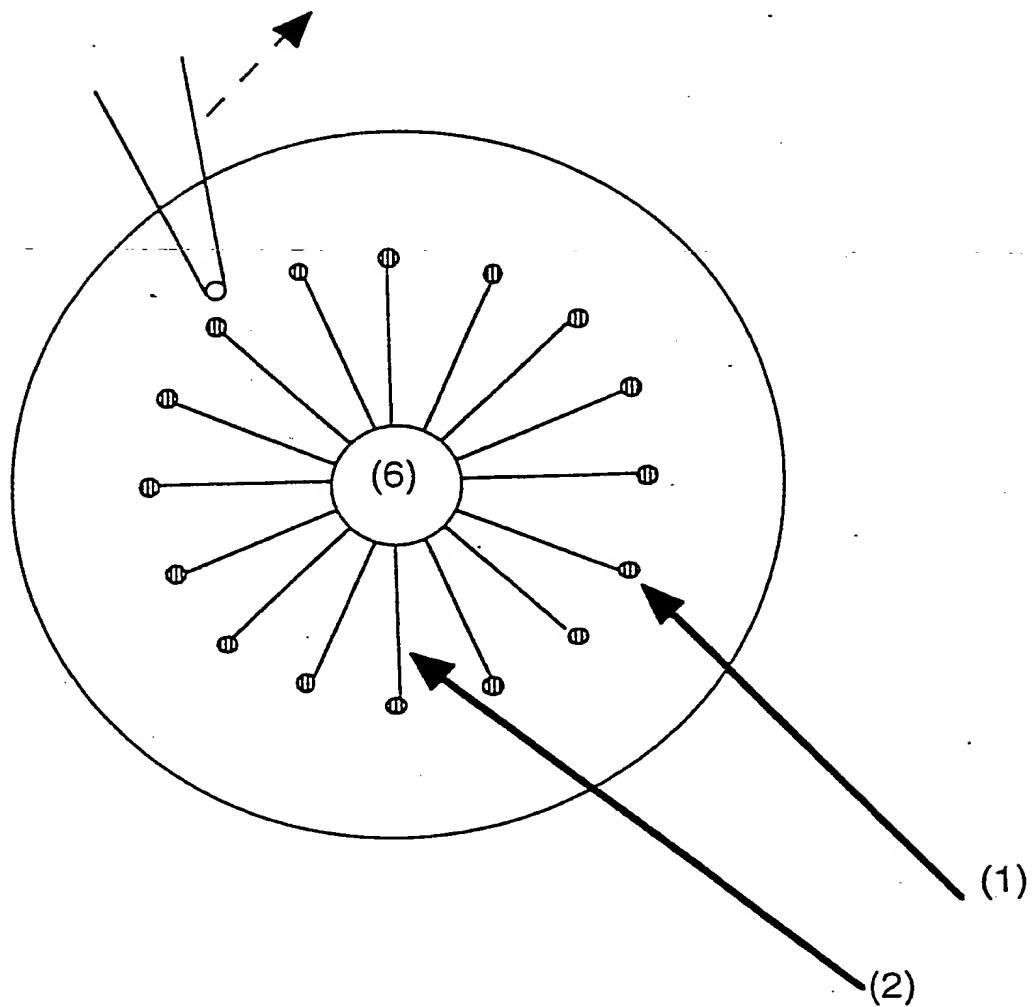
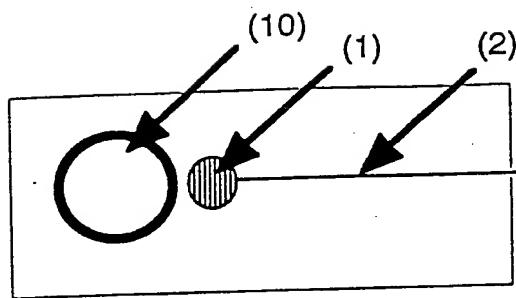
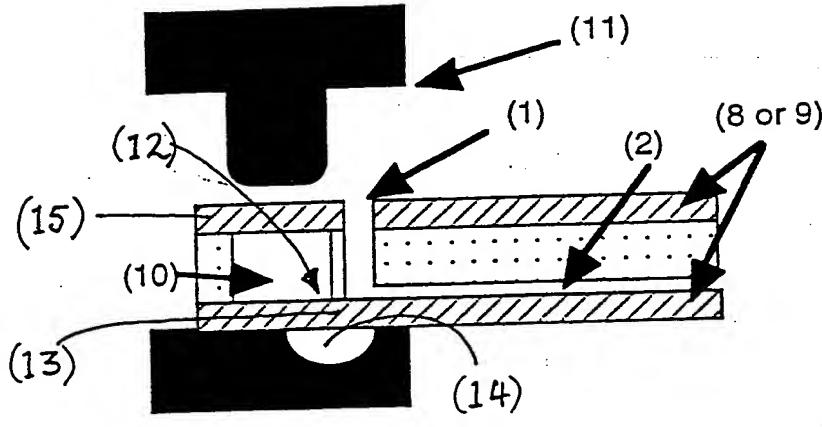


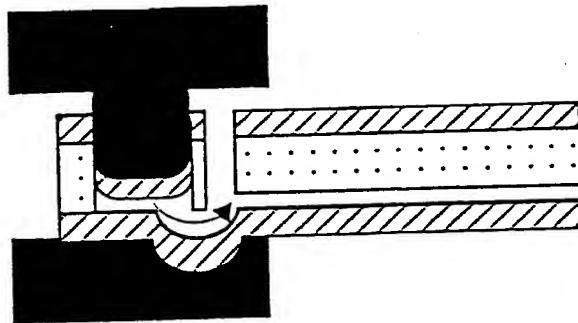
FIG 4

FIG 5a

Top view of a reservoir (10) placed close to a connector (1)

FIG 5b

Cross section of a reservoir (10) placed close to a connector (1)



Opening of the valve under the piston (11) pressure

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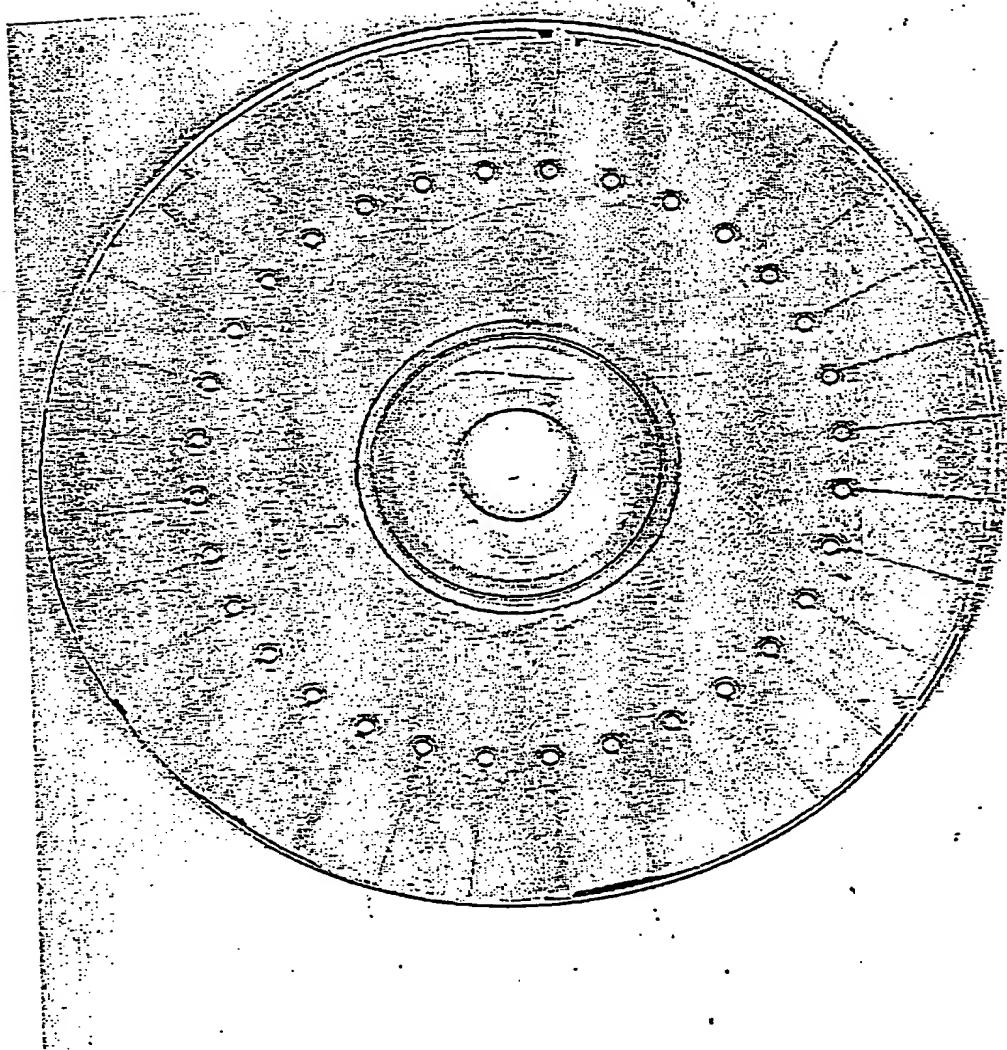


FIG 6

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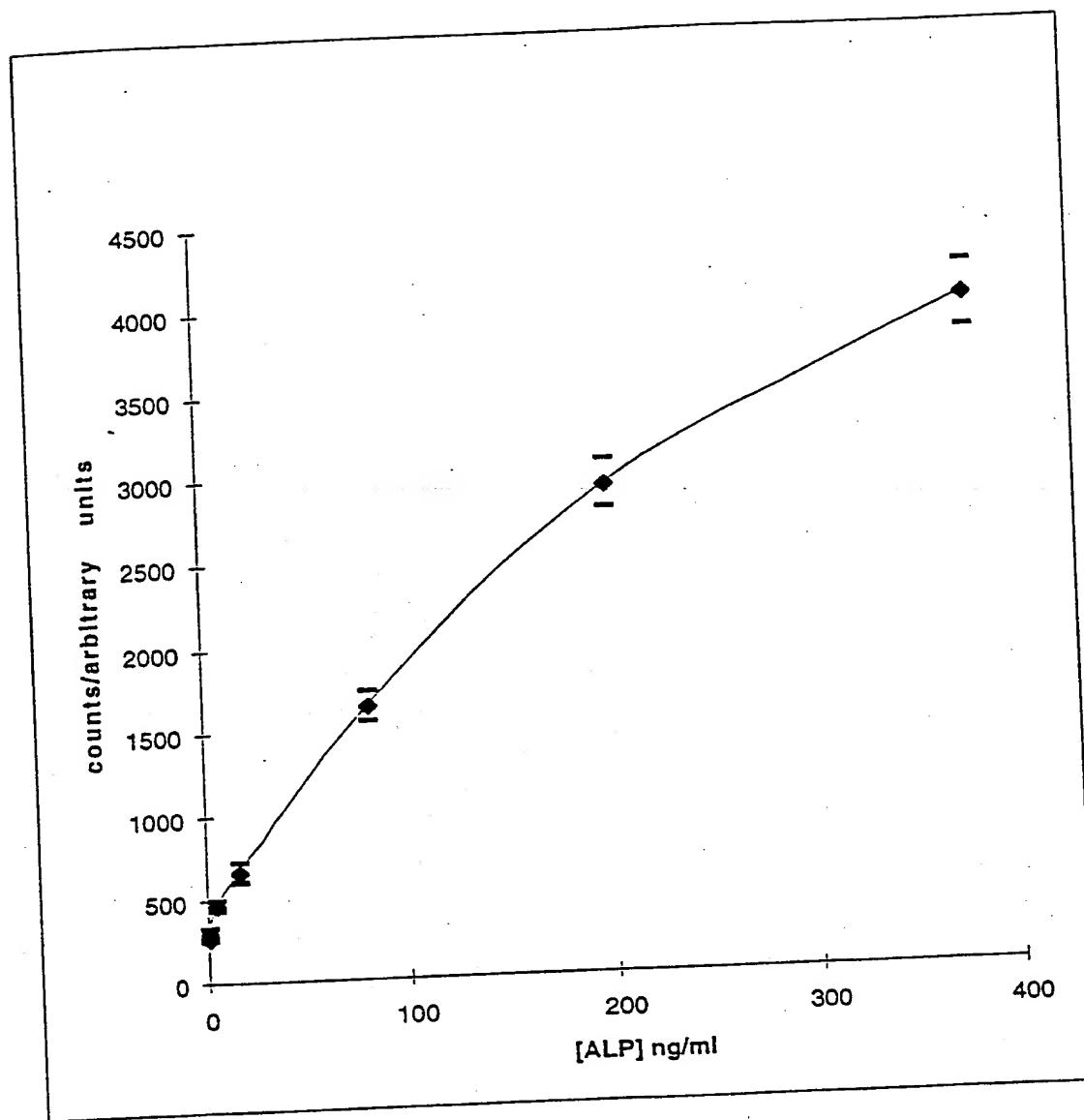


FIG 7

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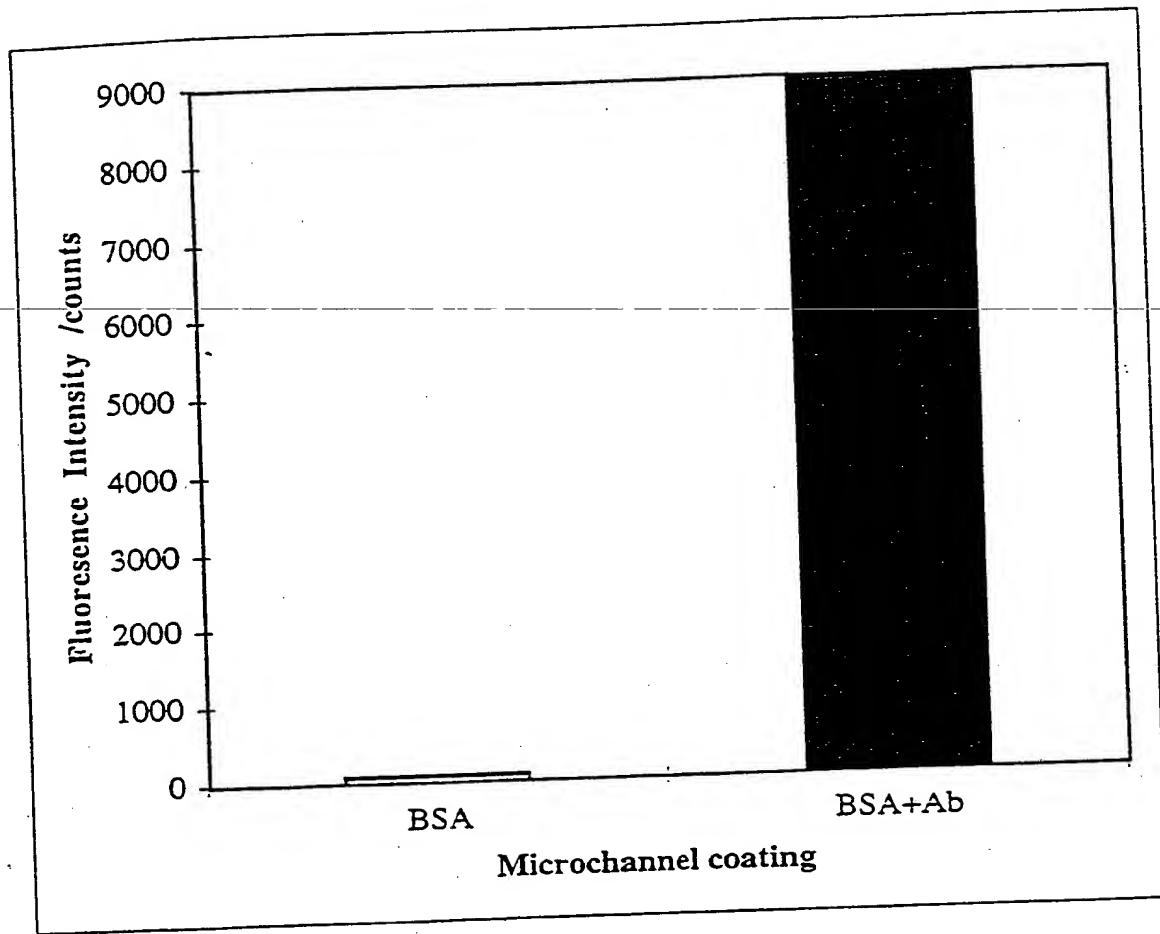


FIG 8

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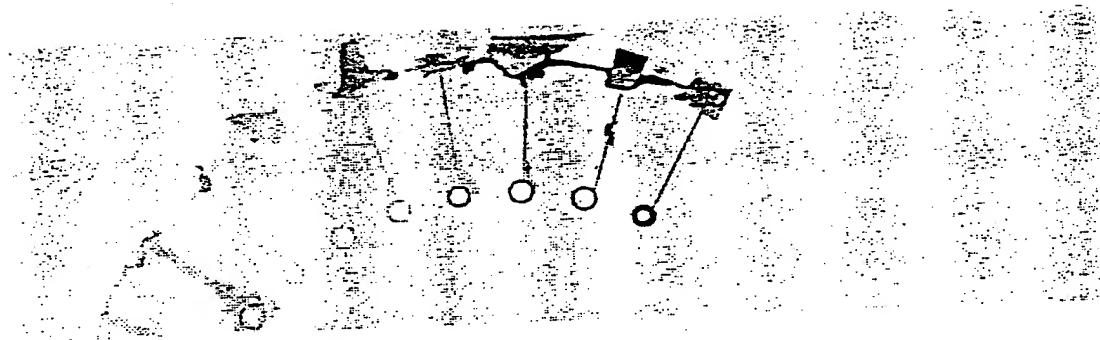


FIG 9

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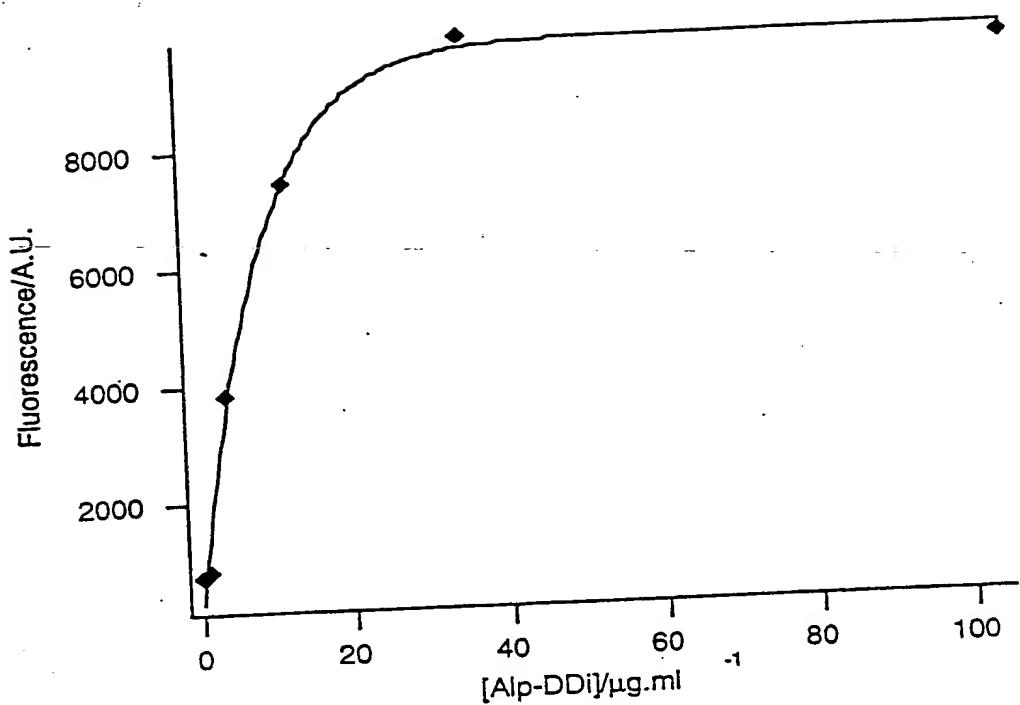


FIG 10

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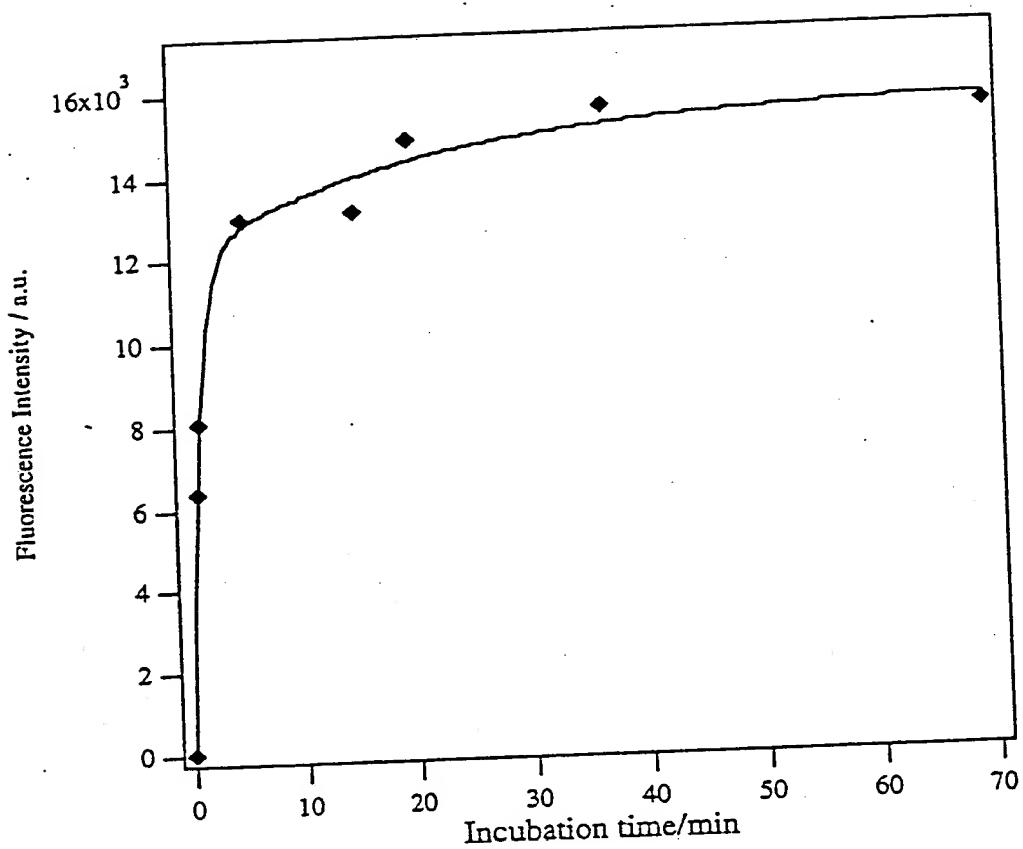


FIG 11

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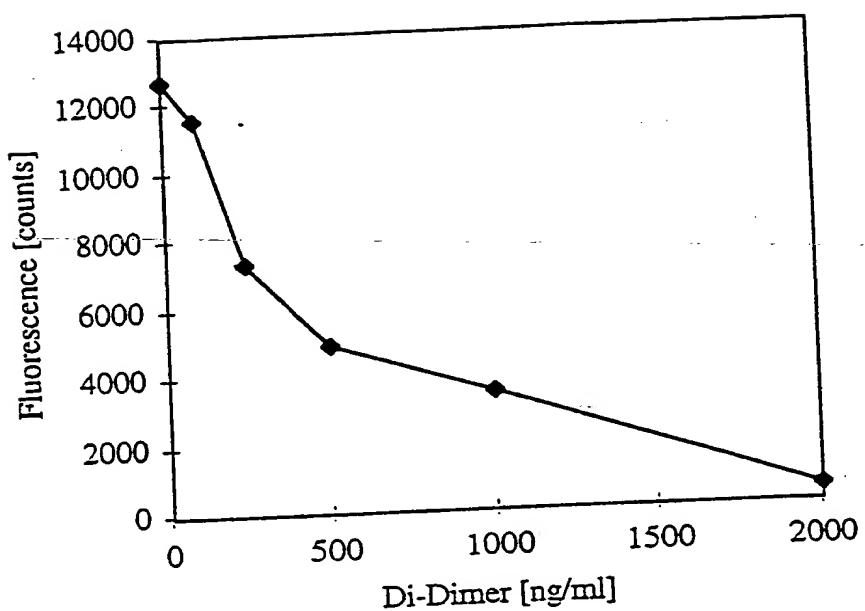


FIG 12